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**Fungal communities are more sensitive indicators to non-extreme soil moisture variations than bacterial communities**

A. Kaisermann<sup>a\*1</sup>, P.A. Maron<sup>b,c</sup>, L. Beaumelle<sup>a</sup>, J.C. Lata<sup>a</sup>

<sup>a</sup> Sorbonne Universités, UPMC Univ Paris 06, UMR 7618, Institut iEES<sub>Paris</sub>, École Normale Supérieure, 46 rue d'Ulm, 75230 Paris Cedex 05, France

<sup>b</sup> INRA, UMR 1347 Agroecology, Dijon, France

<sup>c</sup> INRA, Plateforme GenoSol, UMR1347 Agroecology, Dijon, France

<sup>1</sup> Present address: Michael smith Building, Faculty of Life Sciences, The University of Manchester, Oxford Road, Manchester M13-9PT, United Kingdom

\*Corresponding author: Tel: + 44 161 275 1484

Aurore Kaisermann: [Aurore.kaisermann@manchester.ac.uk](mailto:Aurore.kaisermann@manchester.ac.uk)

Pierre-Alain Maron: [pamaron@diijon.inra.fr](mailto:pamaron@diijon.inra.fr)

Léa Beaumelle: [Lea.Beaumelle@versailles.inra.fr](mailto:Lea.Beaumelle@versailles.inra.fr)

Jean-Christophe Lata: [lata@biologie.ens.fr](mailto:lata@biologie.ens.fr)

## **Abstract**

Many studies have focused on the impact of intense drought and rain events on soil functioning and diversity, but little attention has been paid to the response of microbial communities to non-extreme soil moisture variations. However, small fluctuations of soil water content represent a common situation that ought to be examined before understanding and deciphering the impact of extreme events. Here, we tested the impact of a decrease in average soil water content and small water content fluctuations in non-extreme conditions on microbial community composition and C mineralisation rate of a temperate meadow soil. Two soil microcosm sets were incubated at high and low constant moisture and a third set was subjected to 4 short dry-wet cycles between these two soil moistures. No robust change in bacterial community composition, molecular microbial biomass, and fungal:bacterial ratio were associated with soil water content change. On the contrary, the fungal community composition rapidly alternated between states corresponding to the high and low levels of soil moisture content. In addition, gross C mineralisation was correlated with soil moisture, with a noteworthy absence of a Birch effect (C over-mineralisation) during the wetting. This study suggests that some fungal populations could coexist by occupying different moisture niches, and high fungal community plasticity would classify them as more sensitive indicators of soil moisture than bacteria. Moreover, under non-stressed conditions, the community composition did not affect metabolic performance so a future decrease in average soil moisture content should not result in a supplemental loss in soil carbon stocks by a Birch effect.

## **Keywords**

Soil moisture fluctuation; ARISA fingerprinting method; C mineralisation; metabolic activity; pore size; moisture niche.

## 1. Introduction

Microbial community structure could play a role in the ability of communities to realize different functions but also to resist environmental disturbance (Torsvik and Øvreås, 2002). In the context of global change, microbial community structure could be modified and therefore impact ecosystem functioning. Soil moisture is one of the major factors influencing microbial community structure (Brockett et al., 2012) and the shift of microbial community structure is suspected to contribute to important pulses in net mineralisation during the rewetting of dry soils (Borken and Matzner, 2009). But where some authors have suggested that periods of soil water restriction and wetting of dry soil affect microbial community structure through induced osmotic stress and resource competition occasioning selective pressure (Fierer et al., 2003; Castro et al., 2010), others reported no change in microbial community structure (Griffiths et al., 2003).

These contradictory findings in the literature may be due to the specificity of studied ecosystems but also to differences in experimental approaches (Borken and Matzner, 2009). On one hand, the assumed change of the microbial community structure is often made from circumstantial evidence (*e.g.* changes in biomass or activity). On the other hand, the effects of drying and wetting are frequently not distinguished, and “drying soil” and “drought” often merged. Climatic models forecast a decrease of average soil moisture and an intensification of extreme events (IPCC, 2007), but it is still uncertain to what extent the soil system can become unbalanced under these perturbations. While many studies have focused on intense water-stress, little attention has been paid to the response of microbial communities in non-extreme conditions to characterise their stability to natural variability (Meier et al., 2008). However, small fluctuations of soil water content in non-extreme conditions represent a

common situation that needs to be explored before understanding and deciphering the impact of extreme events.

A limited decrease in soil moisture may be a stressful process for some microorganisms, due to physical constraints that affect bacterial or fungal habitats (Or et al., 2007). At the scale of soil aggregates, the basic units of microbial habitats, drying is heterogeneous and can induce a localized drought that stresses microorganisms; particularly in larger pores (Ruamps et al., 2011). Besides, the decrease of pore connectivity could also modify the microbial community structure by decreasing bacterial mobility and the rate of substrate diffusion (Carson et al., 2010). Fungal communities, on the other hand, are thought to be better adapted to drying than bacteria thanks to hyphal networks which facilitate access to water and nutrients. The question therefore arises as to how fluctuating water content without total drought influences microbial community structure and if the sensitivity of bacterial and fungal community is the same. Beyond the fact that it is still unclear whether a microbial community is associated with a given soil water content, it could be unaltered or alternate between states reflecting the different levels of soil moisture, but also experience another state, reflecting a transient community associated with drying or wetting or both.

The objectives of this study were to examine in non-extreme moisture conditions: (i) whether the composition of bacterial and fungal communities is similar at two contrasting water contents; (ii) how the microbial community composition responds to water content fluctuation within a narrow range of soil moisture; and (iii) whether the microbial community composition can contribute to explain soil functioning. We performed a microcosm experiment consisting of two sets of microcosms kept steadily wet at 64% and 33% Water-Holding Capacity (WHC), respectively. A third set was subjected to 4 dry-wet cycles over a one-month period and subsequently kept steadily at 64% WHC for 4 additional months. At several incubation times, the soil bacterial and fungal communities were characterised by

molecular tools (crude DNA quantity for molecular microbial biomass, A-RISA fingerprinting method for structure and qPCR for abundance), and C mineralisation rate was measured through CO<sub>2</sub> quantification.

## **2. Materials and methods**

### **2.1. Site description and soil characteristics**

Soil was sampled from uncultivated meadow bordering cultural field at Versailles, France (mean annual precipitation: 630 mm; mean annual temperature: 10.5°C); which is an ecosystem with a high ecological importance in urban locations (Manninen et al., 2010). Vegetation (ruderal nitrophilous dominated by *Trifolium repens*) and soil properties of this site are representative of the northern region of France. Fourteen random samples of one kilogram were collected from the topsoil (0-6 cm depth) in August 2010 then combined and homogenized in order to obtain one unique microbial community representative of this ecosystem by lessening spatial heterogeneity. The soil was air-dried, sieved to 4 mm, and vegetation debris, rocks and any fauna visible to the naked-eye were removed before use. The soil is classified as a silty loam (Eutric Cambisol, WRB) with 148 g.kg<sup>-1</sup> of clay, 347 g.kg<sup>-1</sup> of silt and 496 g.kg<sup>-1</sup> of sand. Characterised according to standard methods ([http://www.lille.inra.fr/las/methodes\\_d\\_analyse/sols](http://www.lille.inra.fr/las/methodes_d_analyse/sols)), the pH<sub>H<sub>2</sub>O</sub> was 6, and there was 20.6 g.kg<sup>-1</sup> of organic C, less than 1 g.kg<sup>-1</sup> of CaCO<sub>3</sub>, 1.6 g.kg<sup>-1</sup> of total N hence a C/N of 12.6. Water-holding capacity (WHC) was of 0.41 g of water per g of dry soil.

### **2.2. Experimental design**

Microcosms, consisting of 40 g dry soil equivalent in 126 mL glass bottles, were pre-incubated for 6 weeks at 18.5°C in the dark at desired moisture in order to stabilize the soil microbial communities. Microcosms of high moisture treatment (HM) were maintained at

64% WHC ( $pF=2.24$ ), corresponding to the maximum expected microbial activity, and microcosms of low moisture treatment (LM) at 33% WHC ( $pF=3.67$ ) by sealing them with parafilm<sup>®</sup>. Fluctuating moisture treatment (FM) consisted of 4 cycles of air-drying for one week from 64% WHC until about 33% WHC then wetting to 64% WHC by addition of sterile Milli-Q water. As moisture levels were monitored gravimetrically throughout the incubation (Fig. 3a), the quantity of added water balanced the quantity of water lost during the drying. Time 0 is the first day of the first drying period, and the rewetting events for FM were at 7, 14, 21 and 28 days. After these four cycles, microcosms were subsequently sealed and incubated for a further 4 months at 64% WHC. Three replicate microcosms per FM treatment were destructively sampled at the end of drying period (“Fluctuating Moisture” at low moisture, FM-l) and two days after wetting (“Fluctuating Moisture” at high moisture, FM-h) for the four cycles, then at 60, 90 and 145 days at constant moisture, resulting in  $n = 33$  FM microcosms. Constantly moist microcosms (HM and LM) were sampled in triplicate at 0, 14, 28, 60, 90 and 145 days, resulting in  $n = 18$  microcosms per treatment. Soil samples were stored at  $-20^{\circ}\text{C}$  until required for microbial analyses.

### **2.3. DNA extraction: molecular microbial biomass**

Total soil DNA was extracted and purified following the GnS-GII procedure, as described in Terrat et al. (2012). The DNA concentration of crude extracts was calculated using a calf thymus standard curve. This soil DNA concentration can be used as a robust indicator of soil microbial biomass (Marstorp et al., 2000) defined by Dequiedt et al. (2011) as the molecular microbial biomass. Crude DNA extracts were purified using PVPP minicolumns (BIORAD, Marne La Coquette, France) and GeneClean Turbo Kit (Q Biogene<sup>®</sup>, Illkirch, France) following the manufacturer’s instructions.

### **2.4. Quantitative PCR: microbial density**

The 16S and 18S ribosomal DNA genes copy numbers were determined by real-time PCR using two pairs of universal primers to estimate bacterial (Primer Gold 341F/515R; López-Gutiérrez et al., 2004) and fungal abundances (FR1/FF390; Vainio and Hantula, 2000) following the procedure described in Chemidlin Prévost-Bouré et al. (2011).

## **2.5. ARISA fingerprinting: genetic structure of microbial community**

Automated Ribosomal Intergenic Spacer Analysis (ARISA), a molecular fingerprinting method (Ranjard et al., 2001), has been used to characterise the genetic structure of bacterial (B-ARISA) and fungal (F-ARISA) communities for all samples except the third dry-wet cycle. ARISA was used since it has been demonstrated to be a high-resolution, robust and highly reproducible technique for evaluating microbial communities change through space and time (Ranjard et al., 2001; Jones et al., 2007). The bacterial ribosomal intergenic spacer region (IGS) and the fungal internal transcribe spacer (ITS) were amplified by PCR using primers S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 and ITS1F/3126T, respectively. Purified PCR products were added to deionized formamide and fragments were resolved on polyacrylamide gels under denaturing conditions as described by Pascault et al. (2010) on a LiCor<sup>®</sup> sequencer (ScienceTec, France).

## **2.6. C mineralisation rate**

C mineralisation was measured as the daily CO<sub>2</sub> gas flux on three replicate microcosms per treatment, the day before the wetting, one hour after the wetting as well as the two consecutive days, then once a week the second month, and finally monthly until the end of incubation. Measurements were carried out using a micro-gas chromatograph as described in Kaisermann et al. (2013).

## **2.7. Statistical analysis**

All statistical analyses were performed using the R software v. 2.13 (R Development Core Team, 2011). The ARISA data were analysed using Non-metric multidimensional



scaling (NMDS) implemented in Vegan package (Oksanen et al., 2008) with Bray-Curtis distance measure to generate the dissimilarity matrices. Optimal MDS configurations were determined using 1000 random starts and the lowest stress values were used. The sample discrimination according to treatment, time, and the interaction was tested through a non-parametric manova using the Adonis function (Vegan package; Oksanen et al., 2008) on Bray-Curtis dissimilarity matrices with 999 permutations. This method partitions distance matrices among sources of variations and uses a permutation test with pseudo-F ratios. A first NMDS (Fig. 1a and Fig. 2a) was performed with all samples with the Adonis analysis including 3 treatments (HM, LM, FM) and 7 sampling times. A second NMDS (Fig. 1b and Fig. 2b) was performed for the first month in order to analyse separately the effect of moisture variation in FM treatment with the Adonis analysis including 4 treatments (HM, LM, FM-h, FM-l) and 4 sampling times. Confidence ellipses at 95% were included in ordinations to examine the variability of samples over “time” in the first NMDS, and for “treatment effect” in the second NMDS. Moreover, when the treatment effect was significant, treatments have been compared in a pairwise fashion with independent Adonis tests in order to detail which treatments were different from the others. As the experimental design has nestedness (time effect), we also specified “strata = time” in the Adonis test so that randomization occurs only within each time point.

The differences between treatments for molecular microbial biomasses and 18S:16S ratios were analysed with a linear mixed effect model using the *lme* function implemented in the *nmle* package (<http://lme4.r-forge.r-project.org/>) where ‘time’ was included as a random effect. Significant differences ( $P < 0.05$ ) were evaluated by ANOVA.

For C mineralisation rates, the dependence of the measurement through time on the same microcosms was integrated with “microcosm ID” as a random effect in the *lme*. When C mineralisation is expressed by  $\text{g water.g soil}^{-1}$ , the two constantly wet treatments (LM and

HM) presented no difference throughout the incubation: the mineralisation rate decreased linearly as a function of time, and the equations describing the relationship in both treatments were not significantly different (treatment effect  $P=0.46$ , treatment\*time effect  $P=0.14$ ; Supplemental data, Fig. S1). Therefore, we used data from LM and HM treatments to determine if the variations of moisture in FM treatment induced a supplemental modification of soil respiration than the one expected due to the only modification of water content. The expected mineralisation rate for LM treatment for a given moisture at a given time was calculated as:  $C_{expected} = (-0.0043T + 0.9307) \times Wc$ ; where  $C_{expected}$  is the expected C mineralisation rate in  $\mu\text{g C-CO}_2.\text{gsoil}^{-1}.\text{days}^{-1}$ ,  $T$  the time in days and  $Wc$  the water content in  $\text{gwater.gsoil}^{-1}$ . To estimate the effect of drying and wetting on C mineralisation, the observed value of FM was compared to the expected value using lme with treatment and time as fixed effects and the significance estimated by ANOVA.

### 3. Results

#### 3.1. Genetic structure of bacterial communities

The non-metric multidimensional scale analysis of all B-ARISA profiles showed changes in bacterial community structure over time but without difference between the different treatments (Fig. 1a). The data taken from the first month (Fig. 1b) confirmed the similarity of the bacterial communities between the constant moisture treatments (HM = LM) (Table 1), between high moisture treatments (HM = FM-h) and between low moisture treatments (LM = FM-l). However, the FM treatment after the drying periods (FM-l) was significantly different from the high moisture treatments (HM and FM-h). Therefore, bacterial community composition was identical between the different water contents but a part of the community was different at the end of drying period.

#### 3.2. Genetic structure of fungal communities

The non-metric multidimensional scale analysis of all F-ARISA profiles showed a strong time effect on fungal community structures, especially for days 60 and 145 and also a treatment effect (Fig. 2a). The pairwise comparison showed that fluctuating moisture treatment (FM) was similar to high and low moisture treatments ( $P=0.171$  for HM and  $P=0.126$  for LM, respectively) when HM and LM fungal communities were significantly different ( $P=0.014$ ).

The data taken from the first month (Fig. 2b) revealed significant differences between all treatments except for the fungal communities of LM and FM-l (Table 1). The statistical difference between HM and FM-h was not confirmed by visual observations of NMDS for times 0 and 14 and actually can only be explained by the high variability of replicates at the time 28 (and corroborated by the strong Time\*Treatment effect ( $P=0.005$ ) when the test is done without strata). Therefore, fungal community structure was different between HM and LM, and alternated in the FM treatment between states corresponding to the high and low levels of soil moisture content.

### **3.3. Microbial molecular biomass, abundance and activity**

Molecular microbial biomass (Fig. 3b) and fungi:bacteria ratio measured as 18S:16S ratio (Fig. 3c) were similar between all treatments ( $P=0.07$  and  $P=0.63$ , respectively). The C mineralisation rate of the HM treatment was higher than the LM treatment (Fig. 3d), but was similar when expressed by  $\text{g water}^{-1}$  (Supplemental data S1). The C mineralisation rate of FM fluctuated between the C mineralisation rates of the HM and LM treatments (“time effect”  $P<0.0001$ ). More precisely, the C mineralisation rates decreased when the soil dried, and became similar to the HM treatment after the wetting. There was no difference between observed values of FM and expected values ( $P=0.46$ ) at all incubation times (“Treatment x time effect”  $P=0.14$ ), showing that moisture variations in FM treatment did not induce a

supplemental modification of soil respiration (*i.e.* over-mineralisation) than the one expected due to the modification of water content.

#### **4. Discussion**

For the two levels of water content for the constant moisture microcosms, the bacterial community compositions were similar whereas the fungal community compositions were distinct. Therefore, only the fungal community presents a specific composition depending on soil moisture. This may reflect the difference of ecological habitat between these two guilds (Chenu et al., 2001). Indeed, fungi could preferentially live in large pores, which are filled at high moisture but empty at low moisture; bacteria in turn would live in smaller pores, better protected against these perturbations. In order to discuss ecological patterns, the overall communities can be examined in terms of populations, or subsets of communities. Therefore, the relative abundance of fungal populations inhabiting the large pores could be decreased in dry conditions while the abundance of other populations could increase at the new air-water interface, which explains the dissimilarity of fungal communities between these two moisture levels. Contrastingly, the lack of difference for the bacterial community is unexpected as different pore or aggregate size classes support distinct bacterial populations (Ruamps et al., 2011; Davinic et al., 2012). This could indicate that bacteria either only inhabit the smallest pores still filled with water at low moisture content, or bacterial populations inhabiting large pores are not impacted when pores are empty at low moisture. The latter could be the result of either (i) the presence of sufficient water on pore walls to ensure favourable living conditions or (ii) that bacterial populations themselves maintain a favourable habitat compliments of their lifestyle strategy, since a lot of soil bacteria are able to live in biofilms, embedded in extracellular polymeric substances (Or et al., 2007).

While constant levels of moisture resulted in unchanged bacterial communities, fluctuations resulted in a small modification of the bacterial community structure during the first month. This may result from population shifts within a small portion of the overall community. Indeed, at the aggregate scale, the drainage of pores may be heterogeneous, and since bacteria are dependent on the water in their immediate vicinity, the bacterial community could be only partially in stressful conditions. A finer estimation of how the dynamics of bacterial populations localized in different microenvironments are affected by water fluctuation (i.e. different aggregate size or preferential flow paths; Bundt et al., 2001) is necessary to determine whether a transient community is associated with drying periods. Nevertheless, for the whole of the bacterial community, the community similarity when the water content is half and the lack of large and permanent changes during the water fluctuation, reinforces the hypotheses that either the whole localised bacterial community is adapted to this water disturbance (Griffiths et al., 2003; Meier et al., 2008) or there is no water-stress at this range of soil moisture (Manzoni et al., 2012) at this scale of community integration.

The water fluctuation induced a strong change in the fungal community structure that alternated between states reflecting the high and low levels of soil moisture. This transient response suggests that all species could survive and coexist in this order of magnitude of moisture but that different fungal populations within the community would dominate at different moisture levels. Therefore, notions of tolerance range and ecological optimum of Shelford's law are illustrated here for fungal communities (Shelford, 1931). Indeed, the success of an organism depends upon fulfilment of various conditions, the growth being optimal when all the factors are in optimal range. But if one factor, here the water, is deficient or in excess, the limits of survival of an organism are approached (deviation of ecological optimum) and so its growth is decreased. This is also consistent with Lennon et al.

(2012) who suggested that some taxa may be able to coexist by partitioning the moisture niche axis. When a moisture shift occurs, a reduction of the activity of dominant fungi adapted to previous moisture content could result in a reduction in competitive ability against other fungal taxa, which will then be able to dominate the community at the new water content (Allison and Treseder, 2008). The rapid observed shift is quite surprising, but could be explained by rapid hyphal turnover (Staddon et al., 2003; de Vries et al., 2009) and the ability of some taxa to grow even in drying periods (Bapiri et al., 2010; Yuste et al., 2011).

Therefore, the drought tolerance of fungal communities often claimed in studies could be explained not only by facilitated nutrient access through hyphal networks, but also by a rapid turnover of populations conferring high plasticity to the community. Moreover, the higher variability within the fungal community composition compared to the bacterial community, already highlighted in field condition (Zumsteg et al., 2013), suggests that fungi might be more sensitive indicators of soil moisture than bacteria in non-extreme conditions. Additionally, since such stability of bacterial communities is not always observed after more intense droughts (Fierer et al., 2003; Castro et al., 2010), our study reinforces the concerns about the functioning and resilience of soils undergoing intense droughts because of a permanent impact on bacterial communities.

At this range of soil moisture, C mineralisation rates were positively correlated to soil water content. Two outcomes can be drawn from this result. First, it is worth noting the absence of C over-mineralisation after the wetting, a phenomenon known as the ‘Birch effect’, which is commonly observed in many ecosystems (Borken and Matzner, 2009). This suggests that rainfall without prior soil drought did not induce additional C loss, which supports the findings of Fischer (2009), that a minimal water content must be reached before wetting to generate this flush. Secondly, as the molecular microbial biomass and abundance were similar between treatments, the soil respiration variations were probably due to changes

in metabolic rates of the overall community. Indeed, it is well documented that when soil water potential decreases, the metabolic activity of some microbial species is decreased (Schimel et al., 2007; Manzoni et al., 2012). However, the activity expressed per gram of water is similar at all the water contents, showing that where there is an activity, the activity rate is preserved. With the assumption that the fungal populations that dominated at low water content have an increased activity (or at least their relative contribution within community activity), metabolic adjustments could occur within the overall community to maintain the same metabolic rate. The lack of a link between composition changes and metabolic activity supports the idea that functional redundancy maintains the metabolic rate in non-extreme moisture conditions. Therefore our study suggests, as already presented by Comte and Del Giorgio (2011) and Baltar et al. (2012) for aquatic ecosystems, that overall metabolic performance of a soil microbial community is determined by environmental drivers and can be achieved through different configurations of community composition.

In conclusion, this study illustrates that, taken as a whole and at this range of moisture, (i) the fungal community composition depends on non-extreme moisture conditions in contrast to the bacterial community possibly due to differences in niche preference, (ii) the moisture fluctuation induces a rapid turnover of fungal populations conferring a high community plasticity; therefore fungal community would be a more sensitive indicator of soil moisture than bacteria, (iii) and the community metabolic performance is determined by environmental drivers, the community showing functional redundancy in non-stressed conditions. Finally, our study showed that a microbial community is adapted to cope with non-extreme moisture variation. This reinforces the need to understand why some studies have shown that microbial communities could be lastingly modified by severe drought and evaluate their consequences on ecosystem functioning. For this purpose, it is essential to estimate critical minimum moisture thresholds for microbial community shifts, and thus

assess the effects of intensity and duration of drying and wetting events on the stability of microbial communities.

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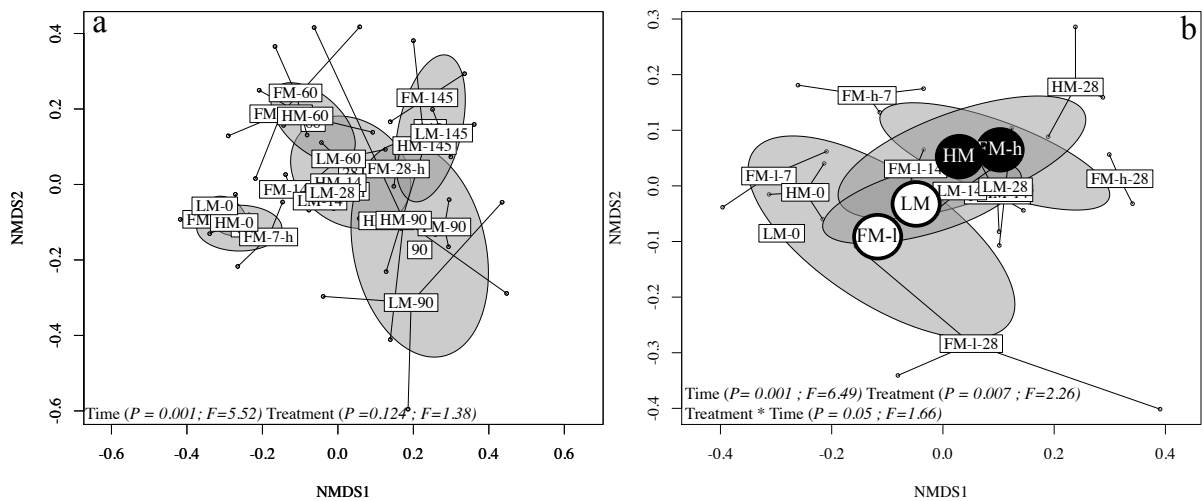
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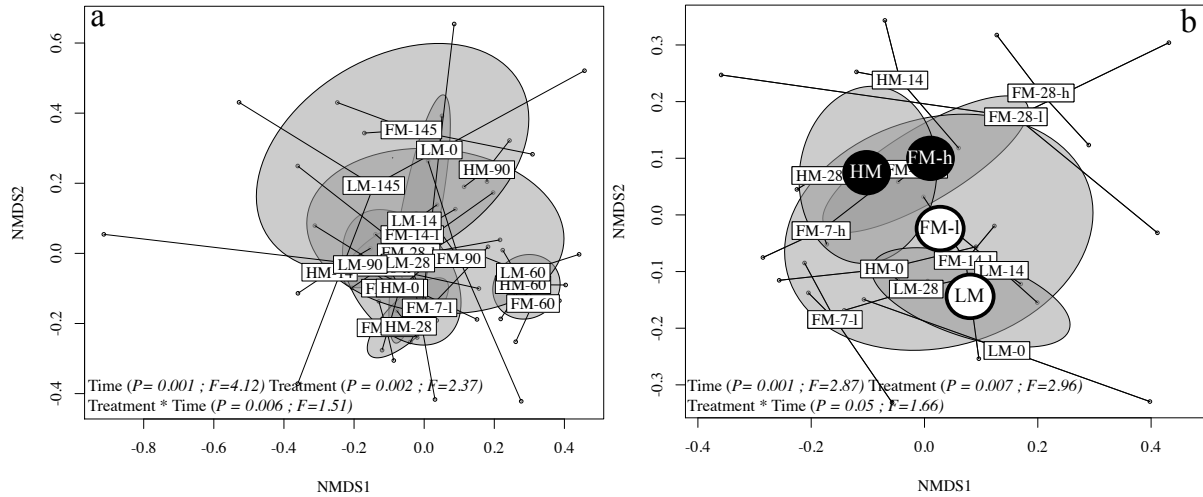
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CAPTIONS

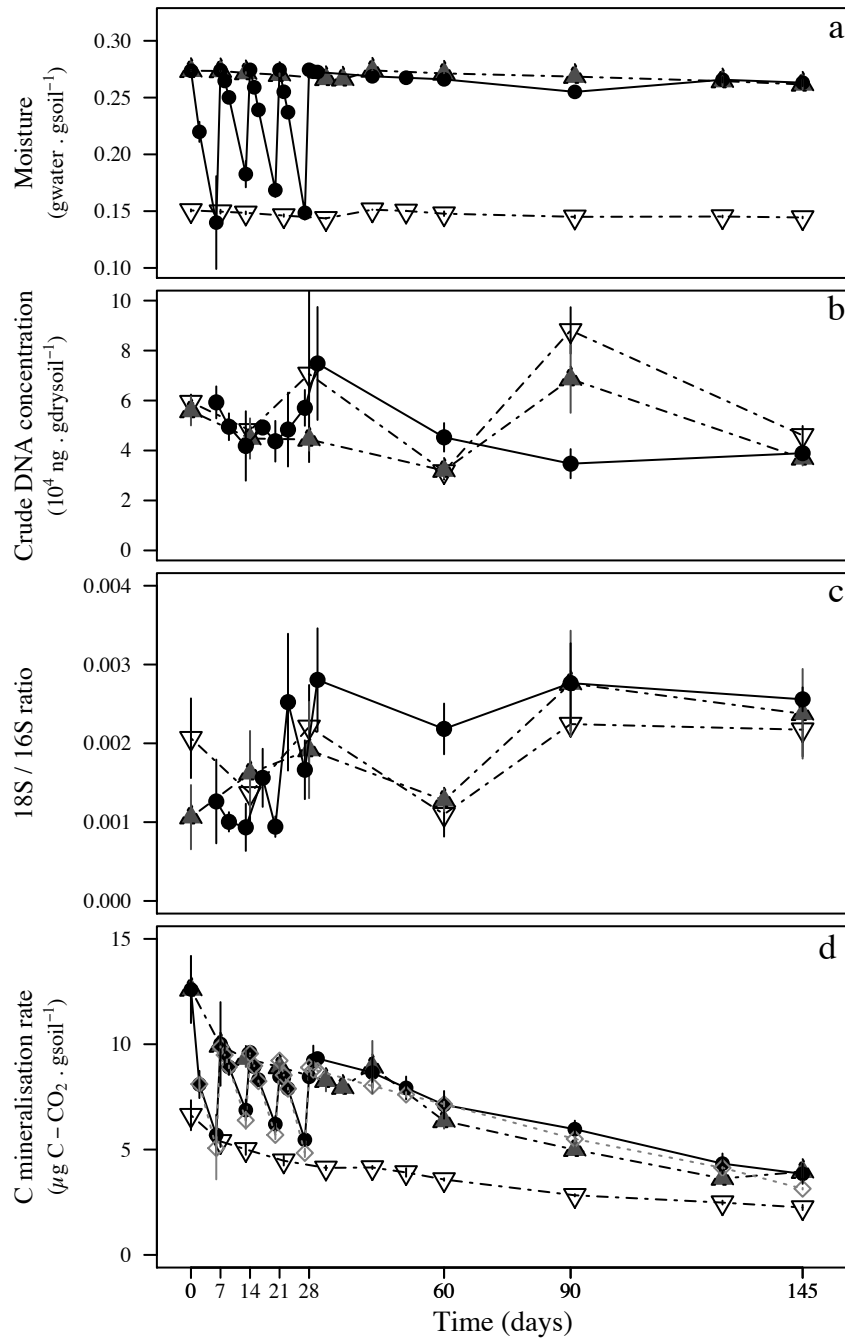
**Table 1.** Summary statistics of pairwise analysis of independent triplicates of bacterial and fungal ARISA profiles for the four treatments High Moisture (HM), Low Moisture (LM) and Fluctuating Moisture at the end of drying periods (FM-l) and two days after the wetting events (FM-h), during the first month of incubation when the moisture fluctuated.



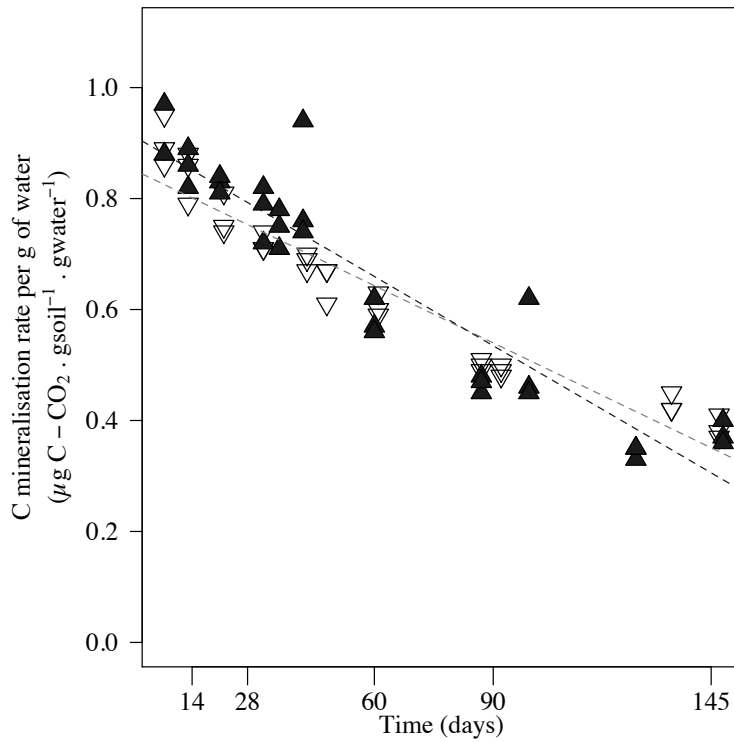
**Fig. 1.** Nonmetric multidimensional scaling analysis generated from independent triplicates of bacterial ARISA profiles for the three treatments High Moisture (HM), Low Moisture (LM) and Fluctuating Moisture (FM) at the end of drying periods (FM-l) and two days after the wetting events (FM-h). Figure (a) shows that the genetic structure of bacterial community from day 0 to day 145 (number indicates the sampling day) is grouped by sampling date. Figure (b) focuses on the first month when the moisture fluctuated, for the first (7), second (14) and fourth (28) dry-wet cycles.



**Fig. 2** Nonmetric multidimensional scaling analysis generated from independent triplicates of fungal ARISA profiles for the three treatments High Moisture (HM), Low Moisture (LM) and Fluctuating Moisture (FM) at the end of drying periods (FM-l) and two days after the wetting events (FM-h). Figure (a) shows the genetic structure of fungal community from day 0 to day 145 (number indicates the sampling day). Figure (b) focuses on the first month when the moisture fluctuated, for the first (7), second (14) and fourth (28) dry-wet cycles.



**Fig. 3.** Moisture (a), molecular microbial biomass (b), fungal:bacterial ratio (c), and C mineralisation rate (d) for the three treatments High Moisture (HM, black triangles pointing upward), Low Moisture (LM, white triangles pointing downward) and Fluctuating Moisture (FM, black circles). Time 0 is the first day of the first drying period for FM treatment. Grey diamonds represent the C mineralisation calculated with linear model for the FM treatment. Data are mean  $\pm$  standard deviation,  $n=3$ .



**S1.** C mineralisation rate expressed per g of water for Low Moisture (LM, white triangles pointing downward) and High Moisture (HM, black triangles pointing upward) treatments. Time 0 is the first day of the first drying period for FM treatment. The dashed lines are the fitted linear model for each treatment (grey and black for LM and HM, respectively). This figure shows that the activity per water unit decrease as a function of time but is similar at the both water content (treatment effect  $P=0.46$ , treatment\*time effect  $P=0.14$ ).